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Hepatocyte growth factor (HGF), also known as Scatter Factor, induces cell growth and cell movement, and is known to promote invasiveness of malignant cells.It also promotes angiogenesis. HGF is known to be produced by fibroblasts within breast tumors, while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves. HGF thus appears to act predominantly as a paracrine factor in breast cancer.High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype, and HGF has been found to be a powerful negative prognostic indicator for breast cancer. Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype and with loss of estrogen-dependent cell growth.Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy.The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy.We will use two approaches for these studies:(1)an antisense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2)a recombinant HGF antagonist molecule (truncated HGF/tHGF) produced in baculovirus and delivered through injection to the peritumoral area.

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Jill M. Siegfried 9-27-99

PI - Signature Date

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Introduction

Hepatocyte growth factor (HGF), also known as Scatter Factor, induces cell growth (1) and cell movement (2), and is known to promote invasiveness of malignant cells (3). It also promotes angiogenesis (4). HGF is known to be produced by fibroblasts within breast tumors (5), while its receptor, the c-Met protein, is expressed on the breast tumor cells themselves (6). HGF thus appears to act predominantly as a paracrine factor in breast cancer (7). High levels of HGF expression within breast tumors correlates with an aggressive tumor phenotype (8), and HGF has been found to be a powerful negative prognostic indicator for breast cancer (9). Expression of the c-Met protein by breast tumors in culture also correlates with an estrogen receptor negative phenotype (10,11) and with loss of estrogen-dependent cell growth. Thus the HGF-c-Met ligand-receptor system may be important in controlling cell growth in breast tumors that have escaped estrogen regulation, a common occurrence in breast cancer patients who have lost responsiveness to anti-estrogen therapy. The hypothesis to be tested in this Idea Grant is that interruption of the HGF-c-Met signaling pathway will inhibit the growth of estrogen-independent human breast cancer cells and could be a useful therapeutic strategy for breast cancer patients who have failed endocrine therapy. We will use two approaches for these studies: (1) an antisense strategy that uses vectors constructed in the U6 RNA expression plasmid and delivered by cationic liposomes and (2) a recombinant HGF antagonist molecule (truncated HGF, tHGF) produced in baculovirus and delivered through injection to the peritumoral area. In vitro experiments examining effects of these agents on breast tumor growth will be followed by in vivo experiments using an s.c. model of tumor growth as well as growth in the mammary fat pad.

Body of Report

This research project was scheduled to start September 1, 1998. The month prior to this, the post-doctoral fellow whom we had recruited to work on this project (Ping Liu, Ph.D.), left the laboratory unexpectedly for personal reasons. Thus the first task to be undertaken was to identify another fellow. Unfortunately this has taken quite some time and a fellow did not join the laboratory until July of 1999. Some work on the project was performed in the meantime by undergraduate students and a technician in the laboratory. However, due to the lack of a dedicated fellow to perform the work, there has been a considerable delay in the progress. However, as discussed with the Program Officer, we are planning to carry over the salary monies that were not spent in the first year and request a no-cost extension at the end of the grant period. In the meantime, in the second year of the grant we hope to make considerable progress on the aims.

Progress to Date on Statement of Work shown in the next section.

1. Produce purified tHGF from baculovirus-infected insect cells.

The recombinant truncated HGF (tHGF) baculovirus stock was provided by Dr. Reza Zarnegar. His laboratory had previously published data indicating this truncated form of HGF acted as a competitive antagonist of HGF (12). High 5 insect cells were infected with the recombinant baculovirus and purified from the culture media using heparin-affinity chromatography. Figure 1 shows a western blot of the 48 kD tHGF molecule. The yield obtained in this first attempt at purification in our hands was only 7μg tHGF/2L of culture media. The yield obtained in other laboratories using this procedure is up to 5mg tHGF/L of culture media. One possibility for the low yield is that the virus titer was very low. In this regard, after infection of the cells with the baculovirus, the cells should be completely lysed by 7 days. This was not observed in our experiments. The cells took up to 4 weeks to lyse. In addition, control cells that were not infected with the baculovirus looked the same as the infected cells throughout the entire culture period. This indicated that the cells died due to lack of feeding during time in culture, not the presence of the virus. This indicated that the virus titer was very low. This could be due to the fact that the virus that we had originally obtained from Dr. Zarnegar was approximately 8 years old. We contacted Dr. Zarnegar to try to obtain a higher titer virus stock or the truncated HGF baculovirus plasmid DNA. Unfortunately, the virus stocks are no longer available and we are still waiting for response regarding the DNA. If we can obtain this plasmid DNA from Zarnegar's laboratory, we can cotransfect High 5 cells with the Bac-N-Blue™ DNA (Invitrogen) and the tHGF plasmid DNA. We can then purifiy recombinant virus from the transfection supernantant using a plaque assay, verify the recombinant plaques by PCR using HGF-specific primers, and generate a high titer stock. Once we obtain a high titer stock, we can infect cells and purify the HGF truncated protein as we had planned. If the tHGF plasmid DNA is no longer available, we can start with the HGF cDNA plasmid and make the truncated HGF baculovirus plasmid form ourselves.

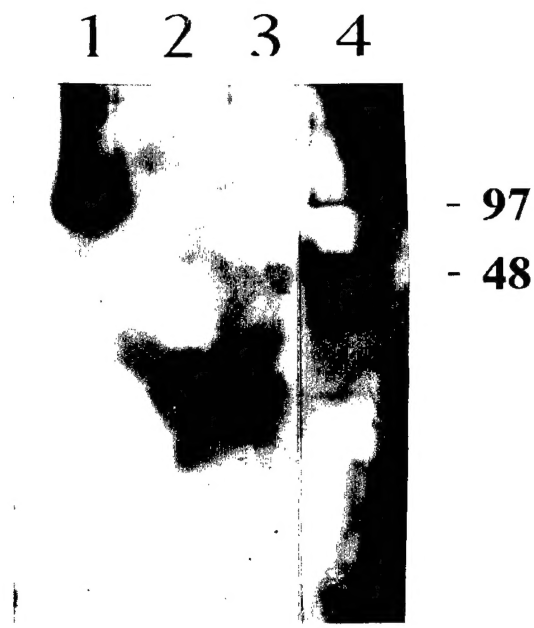

Figure 1. Western blot showing the recombinant full-length 97 kD HGF protein (lane 1) and the truncated 48 kD tHGF (lane 4), isolated from conditioned medium from insect cells infected with the baculovirus tHGF vector. Lanes 2 and 3 contain no loaded protein.

2. Produce sense and antisense constructs for c-Met and HGF and verify sequence.

The sense and antisense constructs for c-Met and HGF have been made and their sequences have been verified using both restriction enzyme analysis and sequencing. The constructs were made by annealing two oligos shown below for each construct and subcloning the annealed oligo into pGEM2U6 (the U6 expression plasmid obtained from L Huang). The primers were designed to contain the XhoI (underlined) and NsiI (double underlined) restriction sites on the ends for subcloning into the same restriction sites in the U6 expression plasmid. These sense and antisense plasmid DNAs have been purified using the Qiagen EndoFree Plasmid Purification Kit. Removal of bacterial endotoxins is known to improve transfection efficiency by up to 50%. The sequences of the sense (S) and antisense (AS) constructs are shown below:

HGF S:

5'-TCGAGATGTGGGTGACCAAACTCCTGCCAGCCCTGCTGCTGCAGCATGCA-3'
3'- CTACACCCACTGGTTTGAGGACGGTCGGGACGACGACGTCGT-5'

HGF-AS:

5'- TATGTGGGTGACCAAACTCCTGCCAGCCCTGCTGCTGCAGCC-3'
3'-ACGTATACACCCACTGGTTTGAGGACGGTCGGGACGACGACGTCGGAGCT-5'

C-MET-S

5'-TCGAGATGAAGGCCCCCGCTGTGCTTGCACCTGGCATCCTCGTGCATGCA-3'
3'- CTACTTCCGGGGGCGACACGAACGTGGACCGTAGGAGCACGT-5'

C-MET-AS

5'- TATGAAGGCCCCCGCTGTGCTTGCACCTGGCATCCTCGTGCC-3'
3'-ACGTATACTTCCGGGGGCGACACGAACGTGGACCGTAGGAGCACGGAGCT-3'

Since HGF is a paracrine factor produced by stromal cells, we intend to target human fibroblasts (obtained as short-term cultures from individual donors) with the HGF vectors and human breast cancer cells with the c-Met vectors.

3. Optimize transfection conditions using breast cancer and human fibroblasts using a reporter gene and liposome preparations.

Transfection conditions have been optimized in the human fibroblasts using green fluorescent protein (pEGFP-N1, Clontech) and different liposome reagents. Our original plan was to use DC-Chol liposomes, which are novel cationic liposomes, described by Gao

and Huang (13). Our belief at the time of the proposal was written was that DC-Chol liposomes would give superior uptake compared to commercially available cationic liposomes. The initial screening of liposome reagents used a constant amount of DNA per plate and a constant ratio of DNA:liposomes. The amount of DNA transfected in the initial screening was 2μg/ 35 mm plate and the ratio of DNA:liposomes was 1:8. We found that LipofectACE and LipofectAMINE gave the best transfection efficiency based the amount of cells that took up the green fluorescent protein versus total cells on the plate (50-60% transfection efficiency). In contrast, DC-Chol gave only 10% transfection efficiency. We next tested different ratios of the DNA:liposome complex to determine the optimum transfection efficiency. Results show that LipofectACE at a 1:10.5 ratio of DNA:liposomes and LipofectAMINE at a 1:15 ratio gave the best efficiency. We chose to use LipofectACE (1:2.5 (w/w) liposome formulation of the cationic lipid dimethyl dioctadecylammonium bromide and dioleoyl phosphtidylethanolamine in water) because there was a considerable amount of cell death using the LipofectAMINE reagent. The same approach will be used to determine the optimal liposome and ratio of DNA:liposomes to be used for each of the breast cancer cell lines. The following Tables summarize the results obtained using the fibroblasts and a representative image is shown in Figure 2.

Table 1:

Normal Human Fibroblasts

Liposome Reagent	*Transfection Efficiency*
Lipofectin	10-20%
LipofectACE	50-60%
LipofectAMINE	40%
LipofectAMINE + Plus Reagent	40-50%
DC-Chol	<10%

Table 2:

LipofectACE		**LipofectAMINE**	
DNA:liposome	*Transfection Efficiency*	*DNA:liposome*	*Transfection Efficiency*
1: 3.5	30%	1: 3	10%
1: 7	40%	1: 5	15%
1: 10.5	55%	1: 10	30%
1: 14	40%	1: 15	50%
1:17.5	High cell death	1: 20	High cell death

Figure 2:

A B C

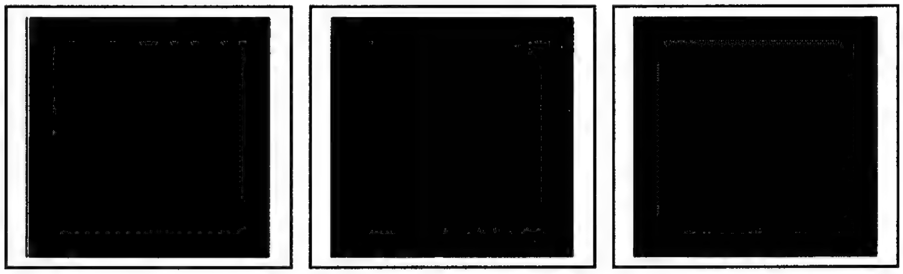

Figure 2. A) Normal human fibroblasts not transfected. B) Normal human fibroblasts transfected with a 1:10.5 ratio of green fluorescent protein to lipofectACE. C) Normal human fibroblasts transfected with a 1:17.5 ratio of green fluorescent protein to lipofectACE.

4. Transfer sense and antisense constructs into breast cancer cells and human fibroblasts and monitor expression of constructs.

We plan to monitor sense and antisense transgene expression and HGF and c-Met endogenous gene expression using a ribonuclease protection assay. Total RNA will be isolated from the cells 48 hr after transient transfection of the sense or antisense constructs. A DNase I digestion of the RNA will be done prior to the ribonuclease protection assay to remove any of the transfected plasmid DNA that may be copurified with the RNA. Protein expression will be monitored 72 hr after transient transfection and will be analyzed by Western analysis.

Probe design for ribonuclease protection assay: 2 probes were designed to detect endogenous gene expression. The first probe, c-Met, was derived from PCR amplification of pMOG (obtained from G. Vande Woude) using primer 1, 5'-CGGAATTCCTTTG CAGCGCGTTGACTTATT-3', and primer 2, 5'-CGCGGATCCTCCTGATCGAGA AACCACAACCTG-3'. Primer 1 contained an EcoRI restriction site at its 5'-end (underlined sequence) for subcloning the PCR amplified product into pSPORT1 (Gibco-BRL) and the remaining sequence is from c-Met. Primer 2 contained a BamHI restriction site at its 5'-end and the rest is the c-Met sequence. The second probe, HGF, was derived from PCR amplification of an HGF cDNA plasmid (obtained from R. Zarnegar) using primer 3, 5'-CGGAATTCAGGCCATGGTGCTATACTCTTGAC-3', and primer 4, 5'-CGCGGATCCTGCCCCTGTAGCCTTCTCCTTGAC-3'. The same restriction sites are present as for the previous probe.

The DNA fragments after PCR amplification will be subcloned into pSPORT1 and the authenticity of the clones will be verified using restriction enzyme analysis. The subclones will be linearized by restriction digestion with EcoRI and used in an in vitro transcription reaction. The resulting antisense RNA probe will be hybridized with total RNA isolated from the cells in the ribonuclease protection assay. The transcripts are designed to be larger

than the protected fragments so that incompletely digested probe can be differentiated from the target signal in a ribonuclease protection assay. The c-Met probe will protect a 146 nt fragment and the HGF probe will detect a 145 nt fragment in the assay.

Five probes were designed to detect transgene expression for HGF-S, HGF-AS, c-Met-S, c-Met-AS and U6 (control for transfection). The DNA templates for in vitro transcription were made by digesting the plasmid DNA for each construct used for transfection with DraI. A 1.5 Kb fragment was isolated in each case which contains the SP6 polymerase promoter sequence and the transcribed portion of each of the constructs. The protected fragment will be 80 nt in each case and will contain portions of the U6 transcribed sequence as well as c-Met or HGF sequence. Thus, we will be able to use a probe for both the endogenous gene and the transgene in the same aliquot of RNA and be able to separate the protected fragments on a gel.

These experiments will be carried out in year 2 of the grant.

5. Perform in vitro experiments with tHGF using human breast cancer cells that do and do not express the c-Met protein

As discussed above, we do not yet have a high enough yield of the tHGF protein to perform these experiments, and since our collaborator, Dr. Zarnegar, is no longer working with tHGF, we may have to re-create the baculovirus vector. This may take some time.

In the meantime, we will continue with the S and AS c-Met constructs and transfer them to breast cancer cells. Based on the literature, there is a correlation between estrogen negative breast cancer cell lines and c-Met expression. We are in the process of verifying this in our laboratory using RT-PCR with the estrogen receptor ERα and ERβ primers and western blotting using ERα and ERβ specfic antibodies, obtained from a commercial source (Santa Cruz Biotechnology). Preliminary experiments with MCF7 cells showed RT-PCR products of the expected sizes for both ERα and ERβ (320 bp and 356 bp). However, the ERα product was stronger. A prominent band was also detected on western blot for ERα and a lesser band for ERβ. We have obtained the breast cancer cell lines T4D7 (reported to be ER positive and c-Met negative), MDA-231 (reported to be ER negative and c-Met positive) and SKBR3 (reported to be ER negative and cMet positive). Because the characterization of these cells lines was done before the discovery of the ERβ, it is possible that some expression of ERβ will be found. Whether or not this affects the c-Met expression is not known. We will be verifying c-Met expression with western blotting by the method we have used previously (9).

After we have characterized these breast cancer cells, we will perform uptake of GFP protein using liposomes to optimize vector delivery and then deliver the S and AS c-Met vectors. We will use a cell line that is negative for c-Met as a control to show no changes in growth after vector transfer.

6. Begin in vivo experiments using tHGF injected into peritumor area of s.c. tumors

Work on this task will be delayed until the second year of the grant, after we show effects of in vitro uptake.

Key Research Accomplishments

1. The cationic liposome LipofectACE gave optimal gene transfer to human fibroblasts, as monitored by green fluorescent protein expression.

2. HGF sense and antisense vectors, as well as c-Met sense and antisense vectors, have been cloned into the U6 RNA expression system. The sequences have been verified, and the vectors target the first 40 bp of the HGF and cMet mRNA, starting at the ATG transcription start site. Plasmid DNA has been purified for optimal gene transfer.

3. An RNAse protection assay strategy has been developed and reagents synthesized to carry out the assay for endogenous and transgene expression in cells transfected with vector DNA encalsulated into cationic liposomes.

4. Assays have been developed to monitor estrogen receptor expression in breast cancer cell lines and to correlate this with c-Met expression.

Reportable Outcomes

We are planning to submit an abstract on use of these vectors in gene transfer to the DOD Breast Cancer Research Program Era of Hope Meeting to be held in Atlanta, Georgia in June of 2000.

Conclusions

Progress on this project was delayed due to loss of personnel from the laboratory. Transfer of DNA vectors into human fibroblasts is feasible using LipofectACE cationic liposomes. Sense and antisense constructs in theU6 RNA have been produced to target the HGF gene in human fibroblasts and the c-Met gene in human breast cancer cells. Methods have been established for monitoring vector expression. Methods have been established for monitoring ER expression and c-Met expression in breast cancer cells.

REFERENCES

(1) Sanders EJ: The roles of epithelial-mesenchymal cell interactions in developmental processes. Biochem. Cell Biol. 66: 530-540, 1988

(2) Stoker M, Gherardi E, Perryman M, Gray J: Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature 327: 239-242, 1987

(3) Rosen EM, Meromsky L, Setter E, Vinter DW, Goldberg ID: Purified scatter factor stimulates epithelial and vascular endothelial cell migration. Proc. Soc. Exp. Biol. Med. 195: 34-43, 1990

(4) Bussolino F, DiRenzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM: Hepoatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J. Cell Biol. 119: 629-641, 1992

(5) Grey AM, Schor AM, Rushton G, Ellis E, Schor SL: Purification of the migration stimulating factor produced by fetal and breast cancer patient fibroblasts. Proc. Natl. Acad. Sci. USA 86: 2438-2442, 1989

(7) Tamashita, J.-i., Ogawa, M., and Beppu, T. Immunoreactive hepatocyte growth factor is present in tissue extracts from human breast cancer but not in conditioned medium of human breast cancer cell lines. Res. Commun. Chem. Pathol. Pharmacol. 82: 249-252, 1993.

(8) Yao, Y., Fuchs, A., Joseph, A., Hastings, H.M., Goldberg, I.D., and Rosen, E.M. Scatter factor protein levels in human breast cancers: clinicopathological and biological correlations. Am. J. Pathol. 149: 1707-1717, 1996.

(9) Yamashita, J.-i., Ogawa, M., Yamashita, S.-i., Nomura, K., Kuramot, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong an independent predictor of recurrence and survival in human breast cancer. Cancer Res. 54: 1630-1633, 1994.

(10) Byers, S., Park, M., Somers, C., and Seslar, S. Breast carcinoma: a collective disorder. Breast Cancer Res. Treatment 31: 203-215, 1994,

(11) Gherardi, E., and Stoker, M. Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. Cancer Cells 3: 227-232, 1991.

(12) Yee, C.J., DeFrances, M.C., Bell, A., Bowen, W., Petersen, B., Michalopoulos, G.K., and Zarnegar, R. Expression and characterization of biologically active human hepatocyte growth factor (HGF) by insect cells infected with HGF-recombinant baculovirus. Biochemistry 32: 7922-7931, 1993.

(13) Gao. X., and Huang, L. A novel cationic liposome regent for efficient transfection of mammalian cells. Biochem. Biophys. Res. Commmen. 179: 280-285, 1991.